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HPLC purification of recombinant NcGRA6 antigen improves enzyme-linked immunosorbent assay for serodiagnosis of bovine neosporosis

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Abstract

The gene for a dense granule protein (NcGRA6) of *Neospora caninum* was expressed in *Escherichia coli* as a His-tag fusion protein and purified by NiNTA affinity chromatography. In a preliminary study, high binding of antibodies from *N. caninum*-negative cows was observed in enzyme-linked immunosorbent assay (ELISA) using NiNTA-purified NcGRA6. Analysis of NiNTA eluates revealed a significant number of *E. coli* proteins that co-purified with recombinant NcGRA6. In an attempt to improve the relative sensitivity and specificity of the NcGRA6-based ELISA, the rNcGRA6 eluates were subjected to a secondary purification using reverse phase-high performance liquid chromatography (RP-HPLC). Analysis of RP-HPLC eluates by SDS-PAGE/silver staining revealed the purification of recombinant NcGRA6 from contaminating *E. coli* proteins. ELISAs using the RP-HPLC purified NcGRA6 (dELISA) or singly purified NcGRA6 (sELISA) for identifying seropositive and seronegative cows in a beef herd experiencing an epidemic outbreak of neosporosis were compared to standard assays based on native tachyzoite protein-immunofluorescence antibody test, immunoblot assay, and ISCOM-ELISA. The relative sensitivity, specificity, and kappa value of the NcGRA6d-ELISA were greatly improved over the NcGRA6s-ELISA when compared to the three native antigen immunoassays. These results indicate that removal of contaminating *E. coli* proteins improves the performance of recombinant NcGRA6 ELISA in diagnosing bovine neosporosis, and may have applicability to the use of recombinant proteins in diagnosing other infectious agents.
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Keywords: *Neospora caninum*; Recombinant protein; Serological assays; Cattle

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1. Introduction

The disease neosporosis is caused by the protozoan *Neospora caninum* and appears to be a major cause of abortion in dairy and beef cattle worldwide. Neosporosis presents a significant problem to veterinary practitioners because there are no obvious clinical signs in the infected dam, aside from abortion or birth of a diseased calf. Although point source (oocyst) infections in naïve cows result in higher rates of reproductive failure than recrudescence of a chronic tissue cyst infection, the most common outcome of both exogenous and endogenous transplacental transmission is the birth of an infected, but otherwise normal calf. Studies on epidemiology of neosporosis and confirmation of clinical pathology in the diagnosis of *N. caninum* as a cause of abortion rely heavily on detecting antibodies in serum or other fluids from adult cows, newborn calves, and aborted fetuses (Jenkins et al., 2000). Serological assay of fetal fluids has been shown to be useful for corroborating histological examination or PCR amplification of *N. caninum* DNA in neural tissue. The presence of antibodies to *N. caninum* in dam serum or in precolostral serum from a newborn calf is indicative of exposure to the parasite. Assays, such as immunofluorescence antibody test (IFAT, Dubey et al., 1988), enzyme-linked immunosorbent assay (ELISA, Paré et al., 1995; Baszler et al., 1996; Björkman et al., 1997; Williams et al., 1997), and immunoblot assay (IB, Schares et al., 1998, 1999) all rely on recognition of a mixture of native *N. caninum* antigens of varying complexity. Several groups have produced recombinant *N. caninum* proteins in *Escherichia coli* or baculovirus as a means of generating sufficient antigen for serodiagnosis (Lally et al., 1996; Louie et al., 1997; Nishikawa et al., 2001; Ahn et al., 2003; Chahan et al., 2003). Most of these recombinant proteins have been engineered to contain a short peptide sequence (e.g., His-tag) to assist in purification. Recent studies in our laboratory (Jenkins, unpublished observations) have shown a high background response to NiNTA-purified NcGRA6 in cows that were negative for *N. caninum* antibodies as indicated by other native antigen-based assays. All attempts to remove these contaminating *E. coli* proteins by extensive washing or alteration of the wash and elution buffers were unsuccessful. The purpose of the present study was to determine if a secondary purification by reverse phase-high performance liquid chromatography (RP-HPLC) could remove the extraneous *E. coli* proteins and thereby increase the usefulness of the recombinant NcGRA6 to assay bovine sera for antibodies to *N. caninum*. A secondary purpose of this study was to compare ISCOM-ELISA, IB assay, and IFAT to each other and to the recombinant NcGRA6-based assays.

2. Materials and methods

2.1. Expression and purification of recombinant NcGRA6

The recombinant NcGRA6 protein was produced in *E. coli* from the pTrcHis expression vector (Invitrogen, Carlsbad, CA) and purified by NiNTA affinity chromatography as described (Lally et al., 1996). Eluates from the NiNTA column were pooled and fractionated using a modification of a reverse phase HPLC method (Fetterer and Barfield, 2003). In brief, the sample was diluted 1:1 in 0.1% trifluroacetic acid (TFA) centrifuged at $20{,}000 \times g$ for 10 min, and applied to a C-4 (Jupiter, 5 μm particle size, 300 ηm pore size, 4.6 mm × 250 mm steel, Phenomenex, Torrance, CA) column. The mobile phase consisted of 0.1% TFA in water (A) and 0.08% TFA, 60% acetonitrile (ACN) and 10% 1-propanol in water (B). The flow rate was 1 ml/min at 22 °C and absorbance was monitored between 210 and 400 nm with a photodiode array detector. The column was equilibrated at 95% A and 5% B, followed by a linear gradient to 50% A, 50% B in 35 min, a linear gradient to 40% A, 60% B at 70 min and a linear gradient to 100% B at 80 min. The mobile phase composition was maintained at 100% B to 85 min. Aliquots (1 ml) were collected and 20 μl of each aliquot dried under vacuum and saved for analysis.

2.2. Protein analysis of NiNTA-purified and HPLC-purified NcGRA6

The protein concentration of recombinant NcGRA6 purified by NiNTA affinity chromatography (singly purified) or RP-HPLC (doubly purified) was estimated using a commercial BCA reagent (Pierce Chemical Co., Rockford, IL). The singly- and doubly-

purified proteins were also analyzed by SDS-PAGE followed by silver staining using a commercial reagent (Pierce Chemical Co.) and by immunoblotting to Immobilon membrane (Millipore, Bedford, MA) followed by immunostaining with mouse anti-His-tag antibodies (Invitrogen).

2.3. rNcGRAenzyme-linked immunosorbent assay

Singly- and doubly-purified recombinant NcGRA6 protein were diluted to 500 ng/ml in 0.05 M sodium carbonate buffer, pH 9.5 and adsorbed to wells of Immulon II microtiter plates (Dynex, Chantilly, VA) for 1 h at 37 °C, then overnight at 4 °C. Unbound NcGRA6 protein was removed and each well was incubated with 5% normal chicken serum for 1 h at RT to block the non-specific binding of antibodies in subsequent steps. The plates were washed twice with PBS containing 0.05% Tween 20 (PBS-TW) and then incubated with sera for 1 h at 37 °C. For purposes of estimating anti-NcGRA6 titers, a pooled positive control bovine serum mixture derived from a neosporosis endemic beef herd was applied at serial two-fold dilutions from 1:100 to 1:1600 in PBS-TW as described (Jenkins et al., 1997). A pooled negative control serum from the same herd was applied in duplicate wells at 1:100 dilution. The experimental sera, derived from a beef herd that was in the midst of a neosporosis abortion outbreak (see below), were applied in duplicate wells at 1:100 dilution. After incubation with bovine sera, the wells were washed three times with PBS-TW, and then incubated for 1 h at RT with alkaline phosphatase-labeled anti-bovine IgG (H + L ch.sp., Kirkegaard-Perry, Gaithersburg, MD). The wells were washed three times with PBS-TW, incubated with 1 mg/ml *p*-nitrophenyl phosphate disodium substrate for 10 min, and read with a Biotek EL312 microplate reader (Biotek Instruments, Winooski, IL) at OD 405. Anti-NcGRA6 titers for all experimental sera were estimated using described procedures (Jenkins et al., 1997).

2.4. Immunofluorescence antibody tests (IFAT-25 and IFAT-100)

All experimental sera, and positive and negative control sera were assayed at both 1:25 (IFAT-25) and 1:100 (IFAT-100) dilution using commercial IFAT reagents (VMRD, Pullman, WA). Using manufacturer's instructions, only sera exhibiting a surface staining pattern were recorded as positive in the IFAT tests.

2.5. ISCOM-ELISA

The experimental sera were assayed by ISCOM-ELISA using described procedures (Björkman et al., 1997; Frössling et al., 2004). Based on previous studies, a cut-off OD value of 0.2 was used to discriminate between *Neospora*-positive and -negative cows.

2.6. Immunoblotting (IB) assay

The experimental sera were assayed by immunoblotting using described procedures (Schares et al., 1998, 2000). A particular serum was considered positive for *N. caninum* if more than one immunodominant band (17, 29, 30, 33, and 37 kDa) was identified in the immunoblot assay.

2.7. Sera

The sera were from 206 adult cows in a beef herd on day 14 of a neosporosis outbreak as described (McAllister et al., 2000; Björkman et al., 2003). In this herd, the reproductive outcome in 43 cows was classified as abnormal (e.g., abortion, or birth of premature or non-viable calf). Analysis by immunohistochemistry or histological examination, or isolation of *N. caninum* from fetal tissue or necropsied calf tissue from 13 cows provided strong evidence for *N. caninum* as the cause of abortion; all 13 cows were negative for other abortifacients (McAllister et al., 2000).

2.8. Statistical analysis

An appropriate cut-off value for the NcGRA6s-ELISA and NcGRA6d-ELISA was determined by TG-ROC analysis using CMDT software (Greiner, 1995) that identifies a cut-off titer at which sensitivity and specificity are equal. TG-ROC analysis utilizes positive and negative reference sera, which in the present study were defined as sera that were positive

in at least three of the standard tests (ISCOM-ELISA, IB assay, IFAT-25, IFAT-100). The NcGRA6-s and NcGRA6-d titers were log transformed for TG-ROC analysis. Sensitivity and specificity were calculated using the software program WinEpiscope (N. de Blas, C. Ortega, K. Frankena, J. Noordhuizen, M. Thrusfield: http://www.clive.ed.ac.uk/winepiscope). The cut-off values for NcGRA6s- and NcGRA6d-ELISAs were used to determine sensitivity and specificity for the recombinant ELISAs relative to the reference standard and the native antigen assays (Von Blumröder et al., 2004). The kappa statistic, as a measure of agreement between NcGRA6s-ELISA or NcGRA6d-ELISA and IFAT, ISCOM-ELISA, or IB was calculated using the WinEpiscope software program.

3. Results

3.1. Purification of NcGRAby RP-HPLC

SDS-PAGE followed by silver staining analysis revealed a number of contaminating proteins with higher and lower relative molecular weight (Mr) in the NiNTA-purified recombinant NcGRA6 protein (rNcGRA6) preparation (Fig. 1). RP-HPLC was capable of separating these proteins from rNcGRA6,

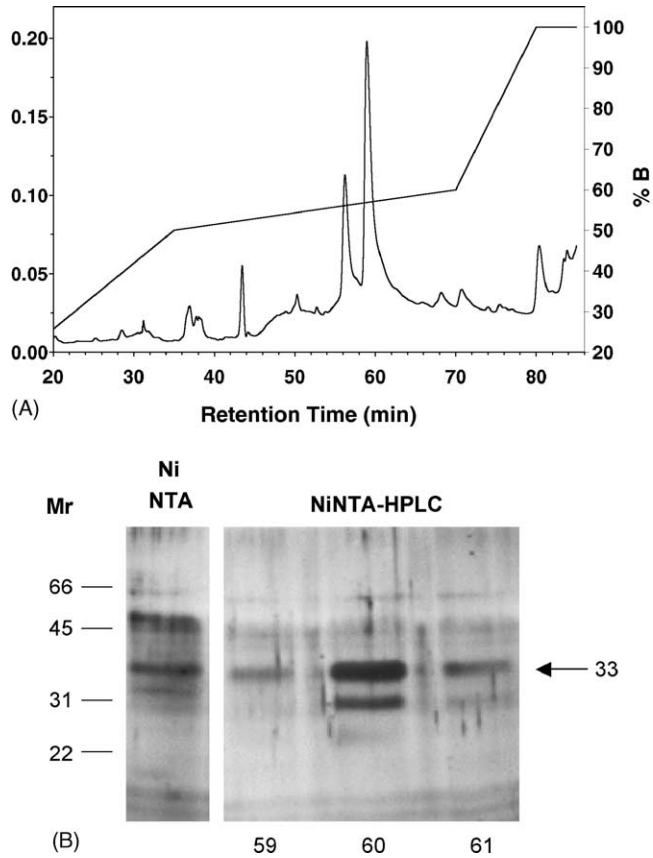

Fig. 1. (A) Chromatographic separation of recombinant NcGRA6 protein mixture. Sample (3 mg) was injected onto the column in about 1 ml of 1:1 mixture of sample with 0.1% trifluoroacetic acid (TFA). The stationary phase consisted of a 4.6 mm × 250 mm steel C4 column. The mobile phase consisted of 0.1% TFA (Buffer A) and 60% ACN. 10% 1-propanol; 0.8% TFA in water (buffer B). The initial conditions were 95% A and 5% B. The column was eluted with a series of linear gradients (left axis) to 100% B at 80 min. Fractions were collected at 1 min (1 ml) intervals and absorbance monitored at 214 nm. (B) SDS-PAGE/silver staining analysis of singly (NiNTA)- or doubly (NiNTA + HPLC)-purified recombinant NcGRA6. The numbers below gel image correspond to eluate fractions. Mr, molecular mass standards.

Table 1
Relative sensitivities, specificities, and kappa values between the native *Neospora caninum* antigen immunoassays-ISCOM-ELISA, immunoblot (IB) assay, immunofluorescence antibody test-25 (IFAT-25), immunofluorescence antibody test-100 (IFAT-100) and a reference standard for detection of antibodies in sera from cows in the midst of a neosporosis outbreak

Standard	Alternative test	Sensitivity (95% CI)	Specificity (95% CI)	Kappa (95% CI)
ISCOM-ELISA	IB assay	98 (95.8–100)	91.1 (83.6–98.5)	0.9 (0.76–1.0)
ISCOM-ELISA	IFA-25	100 (100–100)	53.6 (40.5–66.6)	0.63 (0.5–0.75)
ISCOM-ELISA	IFA-100	99.3 (98.0–100)	73.2 (61.6–84.8)	0.79 (0.65–0.92)
IB assay	ISCOM	96.7 (93.9–99.6)	94.4 (88.3–100)	0.9 (0.76–1.0)
IB assay	IFA-25	99.3 (98.1–100)	53.7 (40.0–67.0)	0.62 (0.49–0.75)
IB assay	IFA-100	99.3 (98.1–100)	75.9 (68.5–87.3)	0.81 (0.68–0.95)
IFA-25	ISCOM-ELISA	85.2 (80–90.5)	100 (100–100)	0.63 (0.5–0.75)
IFA-25	IB assay	85.9 (80.6–91)	96.7 (90.2–100)	0.62 (0.49–0.75)
IFA-25	IFA-100	93.2 (89.5–96.9)	100 (100–100)	0.8 (0.67–0.9.3)
IFA-100	ISCOM-ELISA	90.9 (86.4–95.3)	97.6 (93–100)	0.79 (0.65–0.92)
IFA-100	IB assay	92.1 (87.9–96.2)	97.6 (93–100)	0.81 (0.67–0.95)
IFA-100	IFA-25	100 (100–100)	71.4 (57.8–85.1)	0.8 (0.67–0.93)
Reference standard	ISCOM-ELISA	97.4 (94.9–99.9)	98.5 (94.1–100)	0.94 (0.80–0.99)
Reference standard	IB assay	98.7 (96.9–100)	98.1 (94.5–100)	0.96 (0.83–1.00)
Reference standard	IFA-25	100 (100–100)	56.6 (43.3–70.0)	0.66 (0.53–0.79)
Reference standard	IFA-100	100 (100–100)	79.3 (68.3–90.2)	0.85 (0.72–0.99)

and concentrating the NcGRA6 protein in three to four fractions, which corresponded to the fractions of peak absorbance in the HPLC chromatogram (Fig. 1).

3.2. Comparison of ISCOM-ELISA, IFAT, and IB assay

The kappa values, as a measure of agreement among the native antigen tests ranged from 0.62 to 0.90 (Table 1). Excellent agreement was observed between the ISCOM ELISA and the IB assay (kappa = 0.90) as well as between the IB assay and the IFAT-100 (kappa = 0.82). The IFAT-25 tested much more sera positive than the other tests, resulting in a moderate agreement with the ISCOM-ELISA (kappa = 0.63) and the IB-assay (kappa = 0.62). A high level of agreement was observed between the IFAT-100 and the IFAT-25 (kappa = 0.80), as well as between the IFAT-100 and the ISCOM-ELISA (kappa = 0.79).

The ISCOM-ELISA, IB assay, and IFAT-100 had a good to excellent agreement with the reference standard (kappa = 0.85–0.96, Table 1). Most of the dams (11/13) that had aborted a *N. caninum*-positive fetus were positive using the reference standard (i.e., positive in at least three assays). The ISCOM-ELISA was negative in four cases and the IB assay in two cases. However, all dams that aborted a *N. caninum*-positive fetus were positive in both the IFAT-25 and IFAT-100.

3.3. TG-ROC analysis

The application of the reference standard in the TG-ROC analysis revealed for the NcGRA6s-ELISA a log(titer) cut-off $d_0 = 2.70$ at which the assay had a relative sensitivity and specificity of theta 0 = 78.8% (Fig. 2). Similar TG-ROC analysis of the NcGRA6d-ELISA revealed a log(titer) cut-off $d_0 = 2.78$ and a theta 0 = 83.3% (Fig. 2). Using these cut-off values both the NcGRA6s-ELISA and the NcGRA6d-ELISA detected 11 of 13 dams aborting an *N. caninum* positive fetus.

3.4. Comparison of NcGRA6s-ELISA and NcGRA6d-ELISA to the reference standard

Relative to the reference standard, the ELISA using doubly purified rNcGRA6 exhibited increased sensitivity and specificity compared to singly purified rNcGRA6 ELISA (Table 2). Relative sensitivity increased from 78.4% to 83.7%; relative specificity increased from 79.3% to 83.0%. Also, the kappa value as a measure of agreement between rNcGRA6-ELISAs and reference standard increased from 0.51

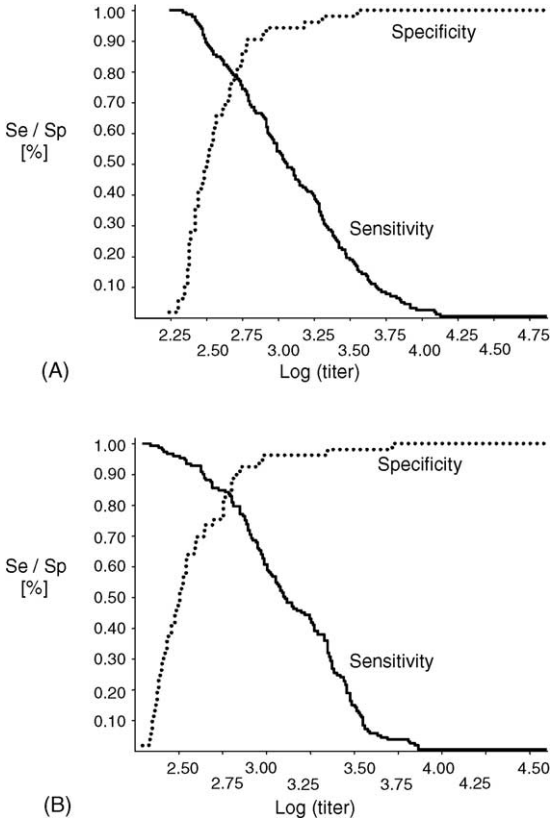

Fig. 2. TG-ROC analysis. (A) A suitable cut-off relative to the reference standard was determined for the NcGRA6s ELISA. When a log (titer) cut-off of 2.70 was applied to classify the results, the ELISA had a relative sensitivity (Se) and a specificity (Sp) of 78.8%. (B) A suitable cut-off relative to the reference standard was determined for the NcGRA6d ELISA. When a log (titer) cut-off of 2.78 was applied to classify the results, the ELISA had a relative sensitivity and a specificity of 83.3%.

to 0.61 when doubly purified rNcGRA6 was used instead of singly purified rNcGRA6 (Table 2).

3.5. Comparison of NcGRA6s-ELISA and NcGRA6d-ELISA to ISCOM-ELISA

ELISA using doubly purified rNcGRA6 exhibited increased sensitivity and specificity compared to singly purified rNcGRA6 ELISA when ISCOM-ELISA was used as the standard assay (Table 2). The relative sensitivity increased from 78.7% to 83.3%; relative specificity increased from 75.4% to 78.6%. Also, the kappa value as a measure of agreement between rNcGRA6-ELISA and ISCOM-ELISA increased from 0.48 to 0.58 when doubly purified rNcGRA6 was used instead of singly purified rNcGRA6 (Table 2).

3.6. Comparison of NcGRA6s-ELISA and NcGRA6d-ELISA to IB assay

Similar increases in relative sensitivity, specificity, and kappa values were observed between the IB assay and rNcGRA6d-ELISA compared to rNcGRA6s-ELISA (Table 2). Relative sensitivity increased from 78.3 to 83.6; relative specificity increased from 74.8% to 81.5%; kappa increased from 0.50 to 0.60.

3.7. Comparison of NcGRA6s- and NcGRA6d-ELISAs to IFAT-25 and IFAT-100

Similar to above comparisons, an increased relative sensitivity (74.4–77.8%) was observed

Table 2
Relative sensitivities, specificities, and kappa values between the rNcGRA6 ELISAs and the native *Neospora caninum* antigen immunoassays (ISCOM-ELISA, immunoblot (IB) assay, immunofluorescence antibody test-25 (IFAT-25), immunofluorescence antibody test-100 (IFAT-100)) and a reference for detection of antibodies in sera from cows in the midst of a neosporosis outbreak

Standard	Alternative test	Sensitivity (95% CI)	Specificity (95% CI)	Kappa (95% CI)
Reference standard	NcGRA6-s	78.4 (71.9–85.0)	79.3 (68.3–90.2)	0.51 (0.37–0.64)
Reference standard	NcGRA6-d	83.7 (77.8–89.5)	83.0 (72.9–93.1)	0.61 (0.47–0.74)
ISCOM-ELISA	NcGRA6-s	78.0 (71.4–84.6)	75.0 (63.7–86.3)	0.48 (0.35–0.61)
ISCOM-ELISA	NcGRA6-d	83.3 (77.4–89.3)	78.6 (67.8–89.3)	0.58 (0.44–0.71)
IB assay	NcGRA6-s	78.3 (71.7–84.8)	77.8 (66.7–88.9)	0.50 (0.37–0.63)
IB assay	NcGRA6-d	83.6 (77.7–89.4)	81.5 (71.1–91.8)	0.60 (0.46–0.73)
IFA-25	NcGRA6-s	74.4 (68.0–80.1)	100 (100–100)	0.46 (0.34–0.57)
IFA-25	NcGRA6-d	77.8 (71.7–84.0)	100 (100–100)	0.51 (0.39–0.62)
IFA-100	NcGRA6-s	76.8 (70.4–83.3)	88.1 (78.3–97.9)	0.50 (0.38–0.63)
IFA-100	NcGRA6-d	81.1 (75.1–87.1)	90.5 (81.6–99.4)	0.58 (0.45–0.71)

between the NcGRA6s- and NcGRA6d-ELISAs when compared to the IFAT-25. The relative specificities of both NcGRA6 ELISAs compared to IFAT-25 was 100%. The level of agreement between the NcGRA6 ELISAs and the IFAT-25 also increased (kappa = 0.45–0.51) when doubly purified NcGRA6 (NcGRA6d-ELISA) was used instead of singly purified NcGRA6 (NcGRA6s-ELISA). Similar increases in relative sensitivity and kappa values were observed when NcGRA6d- and NcGRA6s-ELISA were compared to IFAT-100 (Table 2).

4. Discussion

The present study indicates that RP-HPLC can separate rNcGRA6 from contaminating *E. coli* proteins. In the peak fraction, the 33 kDa NcGRA6 protein and a 31 kDa protein was observed. The relationship between these two proteins is unknown, but it is possible that the latter is a breakdown product of the 33 kDa protein. The study also demonstrated that subjecting NiNTA-purified recombinant NcGRA6 protein to a second purification step increases the sensitivity and specificity relative to standard native antigen immunoassays for detecting antibodies in bovine serum to *N. caninum*. These improvements were probably due to the lower number of false positive reactions with HPLC-purified rNcGRA6 caused by the binding of contaminating *E. coli* proteins in singly (NiNTA) purified NcGRA6 by antibodies normally present in serum against *E. coli*. Previous studies in our laboratory revealed that higher and lower Mr proteins in NiNTA-purified NcGRA6 or non-recombinant protein react with serum from cows that are negative for *N. caninum* by other serological assays (Jenkins, unpublished observations).

In this study, ISCOM-ELISA and IB assay exhibited the highest level of agreement (kappa = 0.90), which explains the similar results when NcGRA6-s and NcGRA6-d ELISAs are compared to either assay. What remains unknown is the appropriate cut-off value for the IFAT. ISCOM-ELISA and IB assay exhibited greater relative sensitivities, specificities, and kappa values with the IFAT-100 compared to the IFAT-25. The rNcGRA6-d ELISA showed higher relative sensitivity and kappa value, but lower relative specificity with IFA-100 compared to IFAT-25. Nearly 30 sera that were negative in ISCOM-ELISA, IB assay, and rNcGRA6 ELISA were also negative in the IFAT-25 indicating the absence of non-specific binding of *N. caninum*-negative cow sera at the lower serum dilution. Another 30 sera that were weakly positive in the IFAT-25 were negative in the IFAT-100 as well as in the other assays. It thus remains unclear whether the IFA-25 is more sensitive than ISCOM-ELISA, IB assay, or rNcGRA6 ELISA or is complicated by non-specific binding at the lower dilution with certain sera.

In conclusion, RP-HPLC purification of rNcGRA6 improved the utility of using this recombinant protein in ELISA for detecting serum antibodies to *N. caninum*. Increased relative sensitivities and specificities, and kappa values were observed with NcGRA6d-ELISA relative to NcGRA6s-ELISA when compared to standard native *N. caninum* antigen assays. The NcGRA6-ELISAs identified as positive 11/13 sera from dams that delivered a confirmed *N. caninum* positive fetus, while four false negative were identified by ISCOM-ELISA and two false negatives identified by IB assay. This apparent high relative sensitivity was at the expense of specificity, which was lower in the NcGRA6d-ELISA compared to ISCOM-ELISA and IB assay. However, the apparent high sensitivities and specificities of ISCOM-ELISA, IB assay, and IFAT-100 relative to the reference standard is due in part to use of native antigen assays to identify ‘‘positive’’ sera. Identifying the reason for the lower sensitivity and specificity in the NcGRA6d-ELISA compared to the native antigen assays will require analyzing a much larger group of cows that are known to be infected with *N. caninum*. The double purification technique (NiNTA followed by RP-HPLC) may also have utility for producing recombinant antigens of other pathogens if absence of contaminating *E. coli* proteins is desired.

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